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Recruitment of Actin Modifiers to TrkA Endosomes Governs Retrograde NGF Signaling and Survival

Anthony W. Harrington,[1,3] Coryse St. Hillaire,[1,3] Larry S. Zweifel,[1,4] Natalia O. Glebova,[1,5] Polyxeni Philippidou,[2,6] Simon Halegoua,[2] and David D. Ginty[1,*]

[1]The Solomon H. Snyder Department of Neuroscience and Howard Hughes Medical Institute, The Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA

[2]Department of Neurobiology and Behavior, Center for Nervous System Disorders and Program in Neuroscience, Stony Brook University, Stony Brook, NY 11794-5230, USA

[3]These authors contributed equally to this work

[4]Present address: Department of Pharmacology, University of Washington Medical Center, Seattle, WA 98195, USA

[5]Present address: Department of Surgery, The Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA

[6]Present address: Howard Hughes Medical Institute, Smilow Neuroscience Program, New York University School of Medicine, New York, NY 10016, USA

*Correspondence: dginty@jhmi.edu

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SUMMARY

The neurotrophins NGF and NT3 collaborate to support development of sympathetic neurons. Although both promote axonal extension via the TrkA receptor, only NGF activates retrograde transport of TrkA endosomes to support neuronal survival. Here, we report that actin depolymerization is essential for initiation of NGF/TrkA endosome trafficking and that a Rac1–cofilin signaling module associated with TrkA early endosomes supports their maturation to retrograde transport-competent endosomes. These actin-regulatory endosomal components are absent from NT3/TrkA endosomes, explaining the failure of NT3 to support retrograde TrkA transport and survival. The inability of NT3 to activate Rac1-GTP–cofilin signaling is likely due to the labile nature of NT3/TrkA complexes within the acidic environment of TrkA early endosomes. Thus, TrkA endosomes associate with actin-modulatory proteins to promote F-actin disassembly, enabling their maturation into transport-competent signaling endosomes. Differential control of this process explains how NGF but not NT3 supports retrograde survival of sympathetic neurons.

INTRODUCTION

Nerve growth factor (NGF), the prototypical member of the neurotrophin family, is essential for development of select populations of neurons in the peripheral nervous system. One of the many functions attributed to NGF is the promotion of sympathetic neuron survival. Indeed, as predicted by the neurotrophic hypothesis, NGF is expressed in target fields of sympathetic neurons and supports their survival via a long-distance signaling mechanism (Levi-Montalcini, 1987). This ultimately ensures that the number of surviving neurons is commensurate with the size and demands of the end organ, a process referred to as "systems matching."

NGF survival signaling is initiated within distal axons and communicated in a retrograde manner to neuronal cell bodies, where it prevents apoptosis. This retrograde signal is activated by NGF binding to its receptor, TrkA, which promotes canonical receptor tyrosine kinase prosurvival signaling (Miller and Kaplan, 2001; Reichardt, 2006), enhanced sensitization to NGF, punishment of neighboring neurons lacking NGF/TrkA signaling, and protection from p75-mediated apoptotic signals (Deppmann et al., 2008). A major mechanism by which this long-distance retrograde signal is carried from distal axons to the cell body is through NGF-induced internalization of NGF/TrkA complexes and the formation of neurotrophin "signaling endosomes." It is now appreciated that TrkA signaling endosomes mediate retrograde control of neuronal survival, growth, gene expression, and synaptogenic signaling events (Barker et al., 2002; Cosker et al., 2008; Howe and Mobley, 2005; Pazyra-Murphy et al., 2009; Sharma et al., 2010). Although several proteins that associate with and function during formation and transport of TrkA signaling endosomes have been identified (Shao et al., 2002; Wan et al., 2008; Wu et al., 2007), proteins that mediate internalization, sorting, axonal transport, signaling, and disassembly of the signaling endosome are largely unknown; therefore, identification of signaling endosome constituents is likely to lend insight into each of these processes.

Although NGF is commonly considered the primary ligand for TrkA, the receptor can also be bound and activated by the related neurotrophin NT3 (Davies et al., 1995). In contrast to NGF's role as a target-derived survival factor, NT3 is produced and secreted by intermediate targets of sympathetic neurons, including the vasculature (Francis et al., 1999). Interestingly, ~50% of sympathetic neurons are lost in *NT3*$^{-/-}$ mice, and those that remain possess shorter and thinner axons than their

wild-type (WT) littermates (Davies et al., 1995; Kuruvilla et al., 2004). This loss of neurons likely reflects the requirement of NT3/TrkA signaling for local axon extension along intermediate targets, a prerequisite for final target-field innervation and acquisition of target-derived NGF, as opposed to a direct effect of NT3 on cell survival. Indeed, although both NGF and NT3 can support TrkA activation and axonal extension (Belliveau et al., 1997; Kuruvilla et al., 2004), NT3 is incapable of eliciting retrograde survival signaling, presumably due to its inability to form retrogradely transported TrkA signaling endosomes. This raises the intriguing question of how NGF and NT3, acting through a common receptor, TrkA, have such divergent functions in the same neuron. Here, we present evidence that target-field-derived growth factor NGF, and not an intermediate-target-derived neurotrophin, controls TrkA endosome maturation through an actin-based endosome-sorting mechanism to support retrograde survival signaling that underlies systems matching.

RESULTS

NGF and NT3 Similarly Activate TrkA, but Only NGF Supports Retrograde TrkA Trafficking and Retrograde Survival in Sympathetic Neurons

Development of postganglionic sympathetic neurons of the superior cervical ganglia (SCG) is critically regulated by two neurotrophins, NT3 and NGF, acting through a common receptor, TrkA (Andres et al., 2008; Belliveau et al., 1997; Davies et al., 1995; Kuruvilla et al., 2004). NT3 supports axonal extension along an intermediate target, the vasculature (Kuruvilla et al., 2004), whereas NGF secreted from final targets is required for target-field innervation, retrograde control of survival, and the formation of synapses between pre- and postganglionic neurons (Glebova and Ginty, 2004; Levi-Montalcini and Booker, 1960; Sharma et al., 2010). Remarkably, although NGF and NT3 can promote both TrkA autophosphorylation and downstream signaling events (Figure 1B), NGF is unique in its ability to promote retrograde TrkA survival signaling to the cell soma (Figure 1A) (Kuruvilla et al., 2004). This is consistent with the notion that the final target but not the intermediate target field defines the amount of survival within a neuronal population. We hypothesized that differences in trafficking of NGF/TrkA and NT3/TrkA complexes within distal axons of sympathetic neurons account for the differences in NGF's and NT3's retrograde signaling capabilities. In order to visualize internalization, sorting, and trafficking of endogenous TrkA in sympathetic neurons, we generated a mouse in which the coding determinants of the Flag epitope tag are knocked into the endogenous *TrkA* locus (Sharma et al., 2010). In cultured SCG neurons dissected from these $TrkA^{Flag}$ knockin mice, Flag-TrkA protein on the cell surface is labeled using an anti-Flag monoclonal antibody (M1), allowing us to monitor the internalization, intracellular trafficking, and subcellular localization of TrkA endosomes. Combining this technique with compartmentalized microfluidic chambers enables an assessment of the formation of TrkA endosomes and their movements from distal axons to cell bodies. As expected, NGF application to distal axons led to internalization and retrograde transport of Flag-TrkA endosomes (Figures 1C and 1D). NT3 also promoted internalization of TrkA when applied to distal axons (Figure 1D) or when applied to cell bodies (Figures S1B and S1C available online). These NT3-formed Flag-$TrkA^{+}$ punctae are likely endosomes as the anti-Flag signal remained following acid/salt stripping, which removes all cell-surface antibody (Sharma et al., 2010). Moreover, NT3/TrkA punctae colocalized with the early endosome marker Rab5 (Figure S2). However, the internalized NT3/TrkA complexes in distal axons failed to undergo retrograde transport to the cell soma (Figure 1C). This is remarkable because both NT3 and NGF promoted comparable levels of TrkA activation and ERK, PLC-γ, and Akt signaling (Figure 1B). Thus, although NGF and NT3 similarly activate TrkA on the axonal surface and promote TrkA-mediated axonal extension and TrkA internalization, these two ligands differ in their abilities to generate transport-competent signaling endosomes and retrograde survival signaling.

A Proteomic Screen for TrkA Endosome-Associated Proteins Reveals an Essential Role for Actin Modulation during Signaling Endosome Maturation and Transport

The mechanisms of signaling endosome formation, sorting, trafficking, and signaling are poorly understood. To address these processes and identify key differences between NGF/TrkA and NT3/TrkA signaling, we performed a proteomic analysis of biochemically isolated TrkA endosomes. The endosome purification procedure was designed to isolate $TrkA^{+}$ early endosomes from a stably transfected PC12 cell line that expresses an epitope-tagged Trk receptor. This purification procedure yielded endosomes with sizes and appearances that were consistent with early endosomes based on electron microscopy (EM) analysis (Figure S3). Protein from purified endosome preparations was extracted and subjected to mass spectroscopy, resulting in the identification of approximately 250 endosome-associated proteins (Table S1). Interestingly, several proteins implicated in actin filament modulation were identified, including actin itself, cofilin, moesin, the Rac1 and Rab5 guanine nucleotide exchange factor (GEF) ALS-2, and a component of the myosin actin motor. The association of several of these proteins with the TrkA endosome was confirmed by immunoblot analysis of TrkA endosome extracted protein (Figure 2A). The presence of a large number of proteins implicated in the control of the actin cytoskeleton led us to focus on its role during TrkA signaling endosome formation, sorting, trafficking, and retrograde transport. Moreover, based on our findings that NT3/TrkA internalizes but fails to initiate long-distance TrkA signaling endosome transport, we hypothesized that differential control of the actin cytoskeleton may represent a point of divergence between NGF/TrkA and NT3/TrkA signaling. Consistent with this idea, NGF and NT3 exhibit dramatic differences in their capacities to evoke the extension of actin-rich filipodial protrusions from their growth cones (Figure S4).

The role of actin during internalization and trafficking of endosomes in yeast is well documented, and its regulation is critical for driving the key sequential steps of invagination, scission, and post-scission movement of endosomes (Robertson et al., 2009). In mammalian cells, a role for actin modulation during endocytosis and endosome trafficking is less clear and may vary depending on cell type and context (Fujimoto et al., 2000).

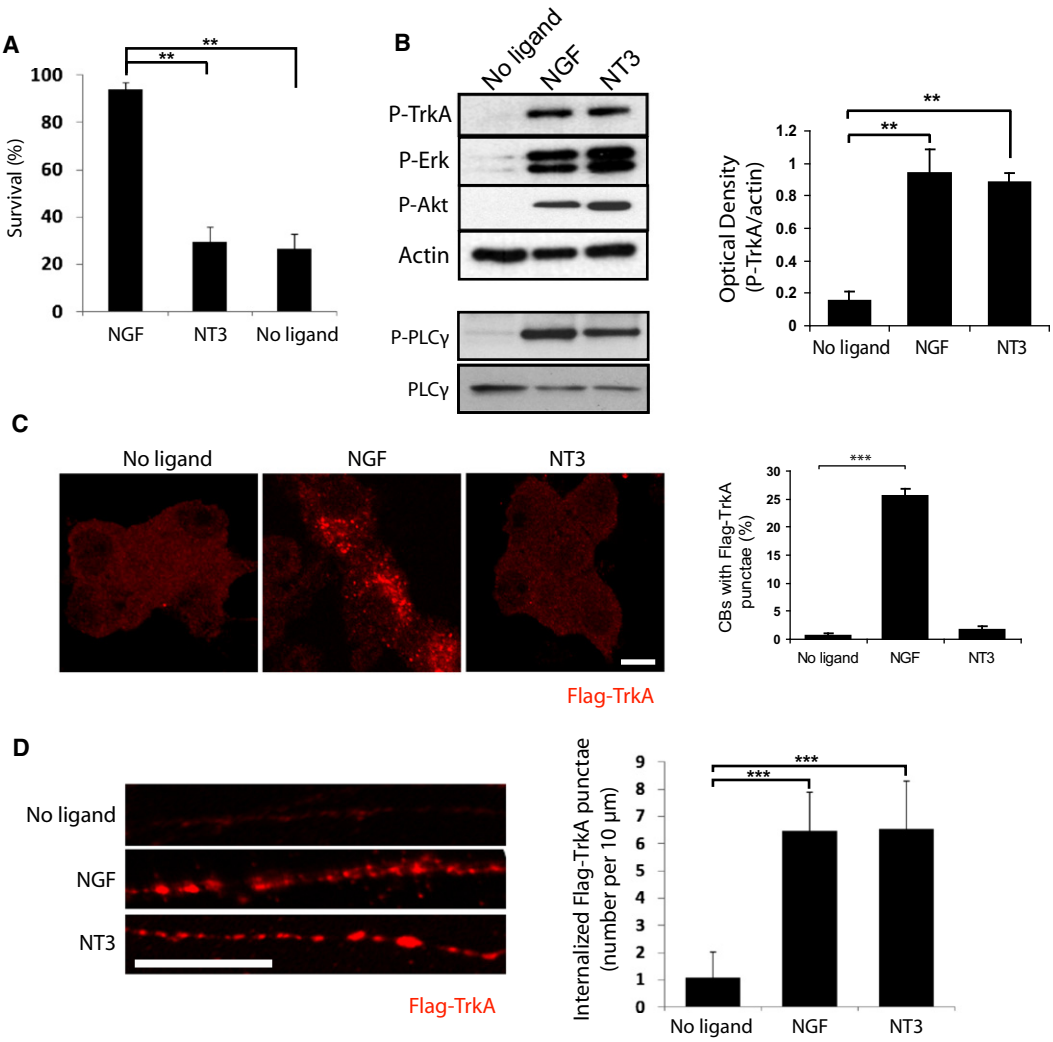

Figure 1. Both NGF and NT3 Activate and Promote Internalization of TrkA, but Only NGF Supports Retrograde TrkA Trafficking and Retrograde Survival

(A) Compartmentalized sympathetic neurons were deprived of NGF for 18 hr, and distal axons were incubated with NGF (100 ng/ml), NT3 (1000 ng/ml), or no neurotrophin. Surviving cells were counted 36–48 hr later.

(B) Mass sympathetic neuron cultures were NGF-deprived overnight and then stimulated with NGF or NT3 for 20 min. Lysates were subject to SDS-PAGE and immunoblotted for the indicated proteins. Upper and lower blots represent different samples.

(C) The Flag-TrkA endosome transport assay was done in compartmentalized sympathetic neurons. The percentage of cell bodies (CBs) containing Flag-TrkA punctae was quantified.

(D) The Flag-TrkA endosome transport assay was performed to assess the extent of internalization of Flag-TrkA and formation of Flag-TrkA endosomes following a 30 min treatment with NGF or NT3 on distal axons of compartmentalized sympathetic neurons. The number of internalized Flag-TrkA endosomes was counted per 10 μm of axon for each condition.

Data in graphs are represented as mean ± standard error of the mean (SEM). Scale bars represent 10 μm. Statistical analysis was done using one-way ANOVA followed by Tukey's post-hoc test. **$p < 0.01$, ***$p < 0.0001$. See also Figure S1 and Figure S2.

We used the actin-modifying drugs Latrunculin A (LatA) and Jasplakinolide (Jasp) to assess the roles of F-actin and actin depolymerization, respectively, during internalization and transport of TrkA endosomes in sympathetic neurons. Surprisingly, the complete loss of F-actin in distal axons or cell bodies and proximal axons of sympathetic neurons exposed to the actin-disrupting compound LatA had no discernable effect on the formation or retrograde transport of TrkA endosomes as assessed by using the Flag-TrkA endosome-monitoring assay (Figure 2B). In contrast, stabilization of the actin cytoskeleton with Jasp led to a complete loss of TrkA endosome retrograde transport, suggesting that F-actin breakdown is required for either formation or transport of TrkA endosomes (Figure 2C). In Jasp-treated distal axons, however, internalization of TrkA and formation of TrkA endosomes were unaffected (Figure 2C, right panels). Thus, actin depolymerization is required within distal axons at

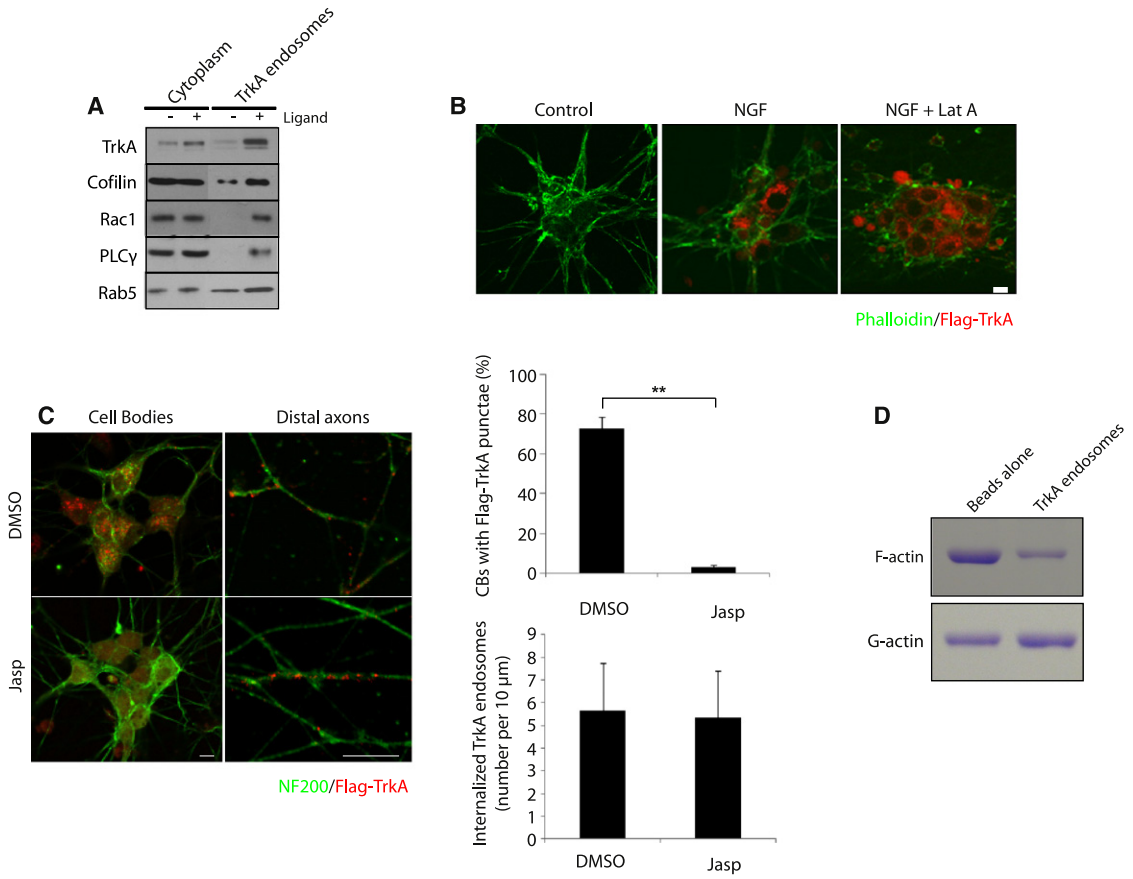

Figure 2. Actin Depolymerization in Distal Axons Is Essential for Retrograde Transport of TrkA Signaling Endosomes

(A) Protein from purified PC12 TrkA endosomes was analyzed by immunoblot.

(B) Sympathetic neurons were grown in microfluidic chambers and pretreated for 30 min with Latrunculin A (LatA) (10 μM) applied to the distal axon chamber. Retrograde transport of TrkA endosomes to cell bodies was assessed using the Flag-TrkA endosome transport assay. Transported Flag-TrkA endosomes are shown in red, and neurons were counterstained with FITC-phalloidin.

(C) Sympathetic neurons grown in microfluidic chambers were treated with Jasplakinolide (10 μM) for 30 min prior to assessment of Flag-TrkA retrograde transport. Internalized and transported Flag-TrkA endosomes are shown in red; neurons were counterstained with NF200. Retrograde signaling endosome transport to the cell body and internalized TrkA in distal axons is quantified (upper and lower right, respectively).

(D) TrkA endosomes are associated with an F-actin depolymerization activity. Control beads or bead-bound Flag-TrkA endosomes were incubated with 2 μM F-actin. After 15 min, beads were removed, F-actin and G-actin were separated by ultracentrifugation, and protein was analyzed by SDS-PAGE and Coomassie staining.

Scale bars represent 10 μm. Graphical data in (C) are represented as mean ± SEM. Statistical analysis was done using the Student's t test. $^{**}p < 0.01$. See also Figure S3, Figure S4, and Table S1.

a stage following TrkA internalization but prior to long-distance microtubule-based retrograde transport of TrkA signaling endosomes.

To determine whether the signaling endosome itself can modulate actin dynamics, we performed an in vitro actin disassembly assay using NGF/TrkA endosomes purified from PC12 cells. Indeed, purified TrkA endosomes accelerated the disassembly of F-actin filaments in vitro (Figure 2D). These results show that TrkA endosomes are associated with actin-modifying proteins, they have an innate ability to promote actin depolymerization, and the depolymerization of filamentous actin is essential for retrograde NGF/TrkA endosome transport at a step following TrkA internalization and the formation of TrkA early endosomes within distal axons.

Actin Depolymerization Mediated by Cofilin Is Required for TrkA Endosome Retrograde Transport and Survival

We next sought to determine which, if any, of the endosome-associated proteins identified in our proteomic screen modulates NGF/TrkA endosome-associated F-actin and whether any of these endosomal components account for the differential capacities of NGF and NT3 to support TrkA endosome retrograde transport. Two prime candidates are cofilin, an actin filament-severing protein, and the small G protein Rac1, which has many and varied effects on actin networks (Hall, 2005; Heasman and Ridley, 2008). Indeed, immunoblot and immunocytochemical analyses revealed that both cofilin and Rac1 are associated with NGF/TrkA endosomes (Figure 2A, Figure 3B, and Figure 4B). Interestingly, experiments aimed at characterizing

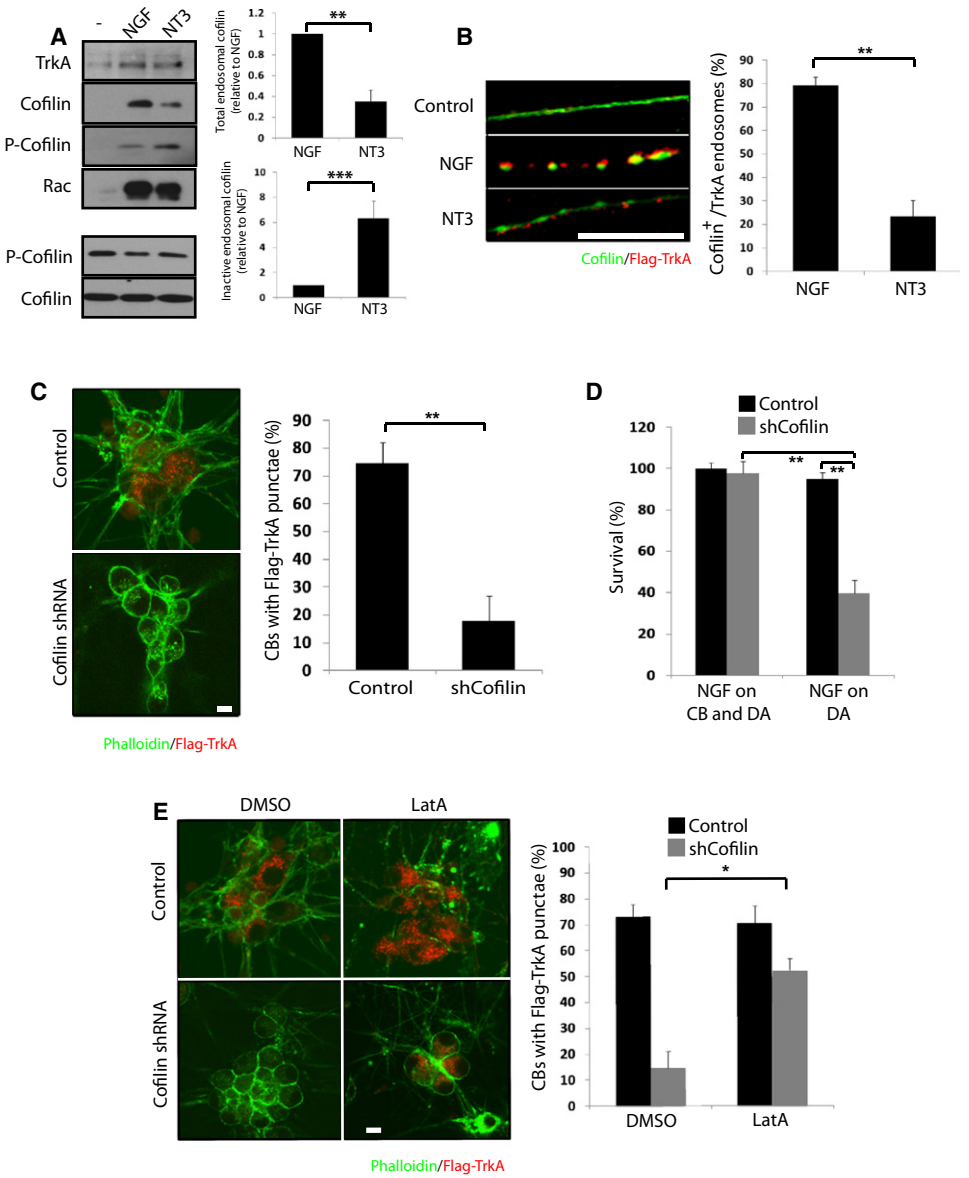

Figure 3. The Actin-Severing Protein Cofilin Is Associated with TrkA Signaling Endosomes and Is Required for Their Retrograde Transport

(A) Early endosomes (top panels) were purified from cultured rat sympathetic neurons deprived of NGF for 18 hr then treated for 20 min with NGF (100 ng/ml) or NT3 (1000 ng/ml) or left untreated. Levels of total cofilin, Ser3-phosphorylated cofilin, and Rac1 were assessed by immunoblot. Relative levels of cofilin and inactive cofilin (Ser3 phosphorylated) associated with NGF and NT3 endosomes were quantified. Whole-cell lysates (bottom panels) from sympathetic neurons treated with NGF and NT3 were analyzed for cofilin and phospho-cofilin.

(B) Colocalization of internalized Flag-TrkA endosomes (red) and cofilin (green) was assessed by immunocytochemistry of distal axons of sympathetic neurons that were untreated or treated with either NGF or NT3.

(C) Lentivirus-infected sympathetic neurons were grown in microfluidic chambers and the retrograde transport of Flag-TrkA endosomes was assessed. Neurons were counterstained with FITC-phalloidin.

(D) The effect of cofilin knockdown on NGF-dependent retrograde survival was assessed for sympathetic neurons grown in microfluidic chambers. Neurons were allowed to project axons into distal chambers and then were infected with either a control lentivirus or a virus expressing an shRNA for cofilin. Survival was monitored 36 hr to 48 hr later under conditions in which NGF was exposed to both cell body and distal axon compartments, or NGF was exposed exclusively to the distal axon compartment.

(E) Inhibition of actin polymerization by LatA rescues the TrkA transport defect in cofilin knockdown neurons. Neurons were treated as in (D) with the addition of a 30 min pretreatment of vehicle or LatA in distal axons prior to performing the Flag-TrkA retrograde transport assay.

Scale bars represent 10 μm. All data in graphs are represented as mean ± SEM. Statistical analysis was done using Student's t test with the exception of that in (D), which was done using one-way ANOVA followed by Tukey's post-hoc test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.0001$.

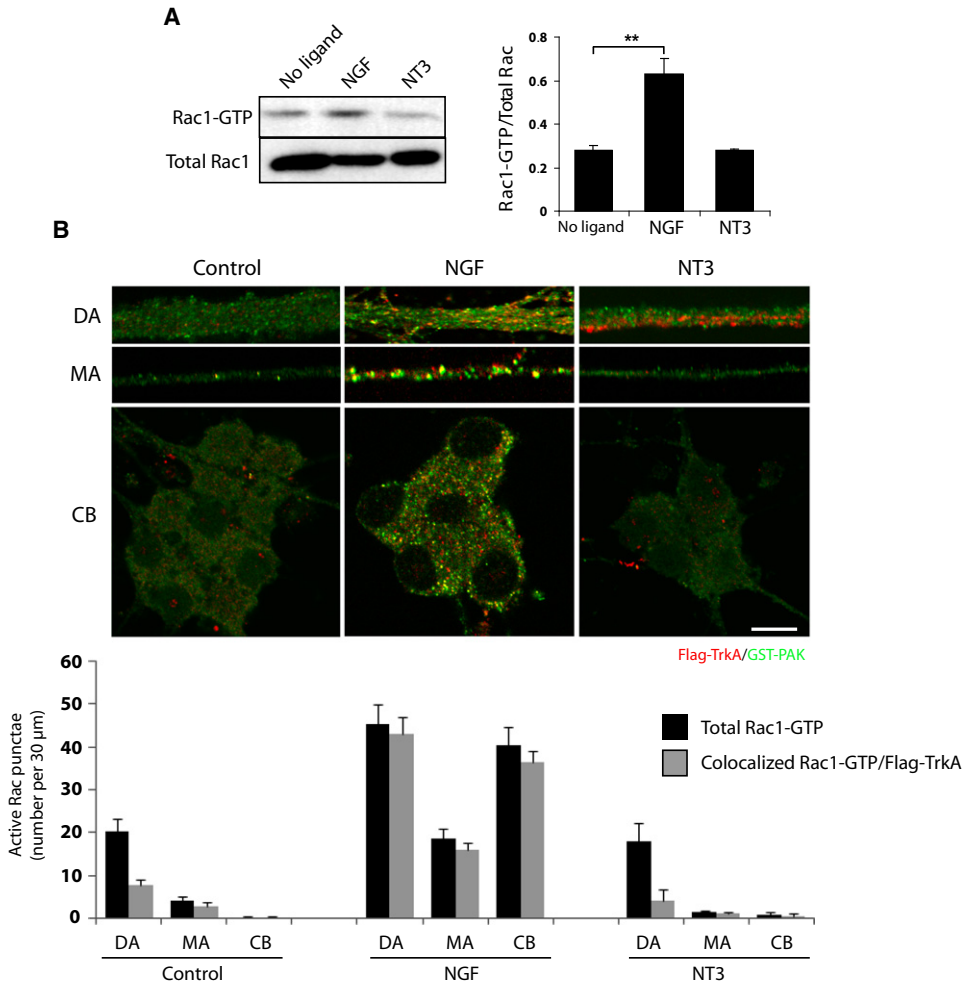

Figure 4. Rac1 Is Activated by NGF but Not NT3 and Is Associated with NGF/TrkA-Containing Endosomes

(A) Dissociated sympathetic neurons were stimulated with NGF (100 ng/ml) or NT3 (1000 ng/ml) for 20 min. Cell lysates were subjected to GST-PAK pull-down assay as a measure of Rac1 activity. Densitometry was performed and signal intensities were normalized against total Rac1. Statistical analysis was done using Student's t test. **$p < 0.01$.

(B) Compartmentalized sympathetic neurons were subjected to the Flag-TrkA endosome assay and distal axons were incubated with vehicle, NGF, or NT3 for 3 hr and then processed with the GST-PAK immunoassay to simultaneously visualize Flag-TrkA (red) and Rac1-GTP (green). Confocal images of distal axons (DA), middle axons (MA), and cell bodies (CB) are shown.

Scale bars represent 10 µm. Quantification of total and colocalized signal is shown below representative pictures. Data in graphs in panels (A) and (B) are represented as mean ± SEM. See also Figure S5 and Figure S6.

the association between cofilin and TrkA endosomes revealed that this association is a defining feature of NGF-formed, as opposed to NT3-formed, TrkA endosomes. In neurons deprived of NGF for 24 hr and then stimulated with NGF for 15 min, cofilin was found to be abundantly associated with early endosome preparations (Figure 3A). In contrast, relatively little cofilin was associated with endosomes harvested from NT3-treated neurons. Moreover, cofilin associated with NGF-formed endosomes was predominantly the active, Serine 3 (Ser3)-unphosphorylated form of the protein, whereas cofilin associated with endosomes following NT3 treatment was more highly phosphorylated on Ser3, which renders it catalytically inactive (Figure 3A). When total phospho-cofilin levels in whole neuron extracts were analyzed, a modest decrease was detected following NGF treatment, whereas NT3 had no effect on cofilin phosphorylation (Figure 3A, lower panels). In addition, although NGF and NT3 treatments led to the formation of comparable amounts of internalized TrkA complexes in distal axons of sympathetic neurons, the majority of NGF/TrkA endosomes were colocalized with cofilin whereas only 23% of NT3/TrkA endosomes colocalized with cofilin (Figure 3B). These findings indicate that a unique ability of NGF is to promote the recruitment of the catalytically active form of the actin-severing protein cofilin to the TrkA signaling endosome.

The finding of an association between cofilin and NGF/TrkA signaling endosomes led us to ask whether this endosome-associated protein plays a role in retrograde TrkA endosome trafficking in axons and retrograde survival signaling. We found

that retrograde transport of NGF/TrkA endosomes from distal axons to cell bodies was dramatically impaired in neurons expressing an shRNA directed against cofilin (Figure 3C), which efficiently knocked down cofilin levels in sympathetic neurons (Figure S4C). Cofilin deficiency did not affect TrkA internalization nor did it alter the extent of TrkA endosome colocalization with the early endosome marker Rab5 (Figure S4B). Moreover, in compartmentalized microfluidic chambers, retrograde survival signaling was compromised in neurons in which cofilin levels were reduced (Figure 3D). In contrast, knockdown of cofilin did not result in death of neurons in which NGF was applied directly to cell bodies (Figure 3D), demonstrating a requirement for cofilin for long-distance TrkA endosome-based survival signaling but not for NGF/TrkA signaling emanating from TrkA on the surface of the soma. Thus, the actin-severing protein cofilin is required for TrkA signaling endosome maturation at a step following internalization and formation of $Rab5^+$ early endosomes but prior to initiation of long-distance retrograde transport.

We next asked whether the loss of cofilin-mediated actin depolymerization was responsible for the defect in retrograde TrkA transport in neurons lacking cofilin. As noted above, exposure of distal axons to LatA, which results in an undetectable amount of filamentous actin within axons (data not shown), does not impair TrkA endosome formation nor does it compromise retrograde TrkA endosome transport (Figure 2B). Remarkably, when added to distal axons of neurons in which cofilin was eliminated, LatA almost completely rescued the deficit in retrograde TrkA endosome transport (Figure 3E), indicating that pharmacological disassembly of F-actin can substitute for cofilin. Thus, although the actin cytoskeleton is dispensable for NGF-dependent internalization of TrkA, cofilin-mediated disassembly of F-actin is essential for retrograde transport of TrkA signaling endosomes from distal axons to the cell body and thus retrograde survival signaling.

Rac1 Is Differentially Activated by NGF and NT3 and Is Associated with NGF/TrkA-Containing Endosomes

Like cofilin, the Rho family GTPase Rac1 was found to be associated with purified TrkA endosomes (Figure 2A and Figure 3A). Moreover, Rac1 is a major regulator of the actin cytoskeleton. Under certain conditions, Rac1 is necessary for cofilin activation through the promotion of dephosphorylation of cofilin Ser3 (Pandey et al., 2009; Sun et al., 2007), and it is implicated in Trk receptor endocytosis and transport (Philippidou et al., 2011; Symons and Rusk, 2003; Valdez et al., 2007). Additionally, regulators of Rac1 activity are implicated in the control of endosomal trafficking (Devon et al., 2006; Sun et al., 2006). Therefore, we next asked whether Rac1 mediates NGF/TrkA signaling endosome formation, maturation, and/or trafficking in sympathetic neurons and if Rac1 signaling controls cofilin activity and/or its association with the TrkA endosome.

We first assessed whether both NGF/TrkA and NT3/TrkA signaling activate the Rac1 signaling pathway in sympathetic neurons or whether, like cofilin, Rac1 activation is more prominently associated with NGF/TrkA. To do this, sympathetic neurons were treated for 20 min with either NGF or NT3, and lysates were subjected to precipitation using GST-PAK, which binds with high affinity to the active, GTP-bound form of Rac1 and its relative Cdc42. Immunoprecipitates were then subjected to immunoblot analysis using an antibody specific for Rac1. Interestingly, although NGF treatment of sympathetic neurons led to an increase in the level of Rac1-GTP, NT3 was unable to activate Rac1 (Figure 4A), despite these neurotrophins triggering comparable amounts of TrkA phosphorylation, activation of the canonical effectors (Figure 1B; Figure S1A), and TrkA internalization (Figure 1D; Figures S1B and S1C). GST-PAK was also used to assess the subcellular distribution of Rac1-GTP in sympathetic neurons that were either untreated or treated with NGF or NT3. These Rac1-GTP localization experiments were done using sympathetic neurons from $TrkA^{Flag}$ mice; double-labeling with the Flag-TrkA endosome-monitoring assay allowed for an assessment of active Rac1 and TrkA endosome colocalization. The GST-PAK immunolabeling assay specifically detects Rac1-GTP in these immunolocalization experiments, as we observed a near complete loss of GST-PAK-binding sites in neurons pretreated with a Rac1 shRNA virus that significantly reduces expression of Rac1 (Figure S5A). Moreover, NGF treatment of distal axons of compartmentalized $TrkA^{Flag}$ sympathetic neurons led to a robust increase in the number of active Rac1-GTP punctae (Figure 4B). Remarkably, Rac1-GTP punctae were observed throughout the neuron, including the entire axon and cell body, and they were colocalized with Flag-TrkA endosomes (Figure 4B). Although NT3 led to a robust accumulation of Flag-TrkA endosomes in distal axons, these NT3/TrkA endosomes were not observed in proximal axons and cell bodies and they were rarely, if ever, associated with Rac1-GTP (Figure 4B). Thus, NGF, but not NT3, activates Rac1 in sympathetic neurons and, as is the case for cofilin, Rac1-GTP is associated with NGF/TrkA endosomes, whereas NT3-formed endosomes are devoid of Rac1-GTP.

Rac1 Is Necessary and Sufficient for Retrograde TrkA Trafficking and Retrograde Survival Signaling

To test whether Rac1 is required for formation or transport of NGF/TrkA endosomes, compartmentalized sympathetic neurons were infected with a virus expressing a dominant-negative form of Rac1 (RacN17), and the Flag-TrkA endosome transport assay was performed following treatment of distal axons with NGF. Although Rac1 was dispensable for NGF-dependent internalization of TrkA (Figure S6A), it is required for retrograde transport of TrkA endosomes (Figures 5A and 5B). This conclusion is supported by experiments that used either a Rac1 shRNA to reduce Rac1 levels or the small-molecule Rac1 inhibitor, EHT 1864 (data not shown), which blocks activation of all Rac family members by maintaining them in their GDP-bound state (Shutes et al., 2007). Moreover, as was observed following inhibition of cofilin, inhibition of Rac1 with RacN17 led to a loss of retrograde survival signaling in neurons in which NGF was added exclusively to distal axons (Figure 5C). Parallel to findings with cofilin inhibition, Rac1 inhibition did not perturb survival of neurons in which NGF was applied directly to cell bodies (data not shown).

We next asked whether NT3's inability to support retrograde transport of TrkA endosomes and retrograde survival signaling is due to its inability to activate Rac1 and form TrkA endosomes that associate with Rac1-GTP. This possibility was tested in experiments in which a constitutively active form of Rac1 (RacV12) was expressed in $TrkA^{Flag}$ sympathetic neurons grown

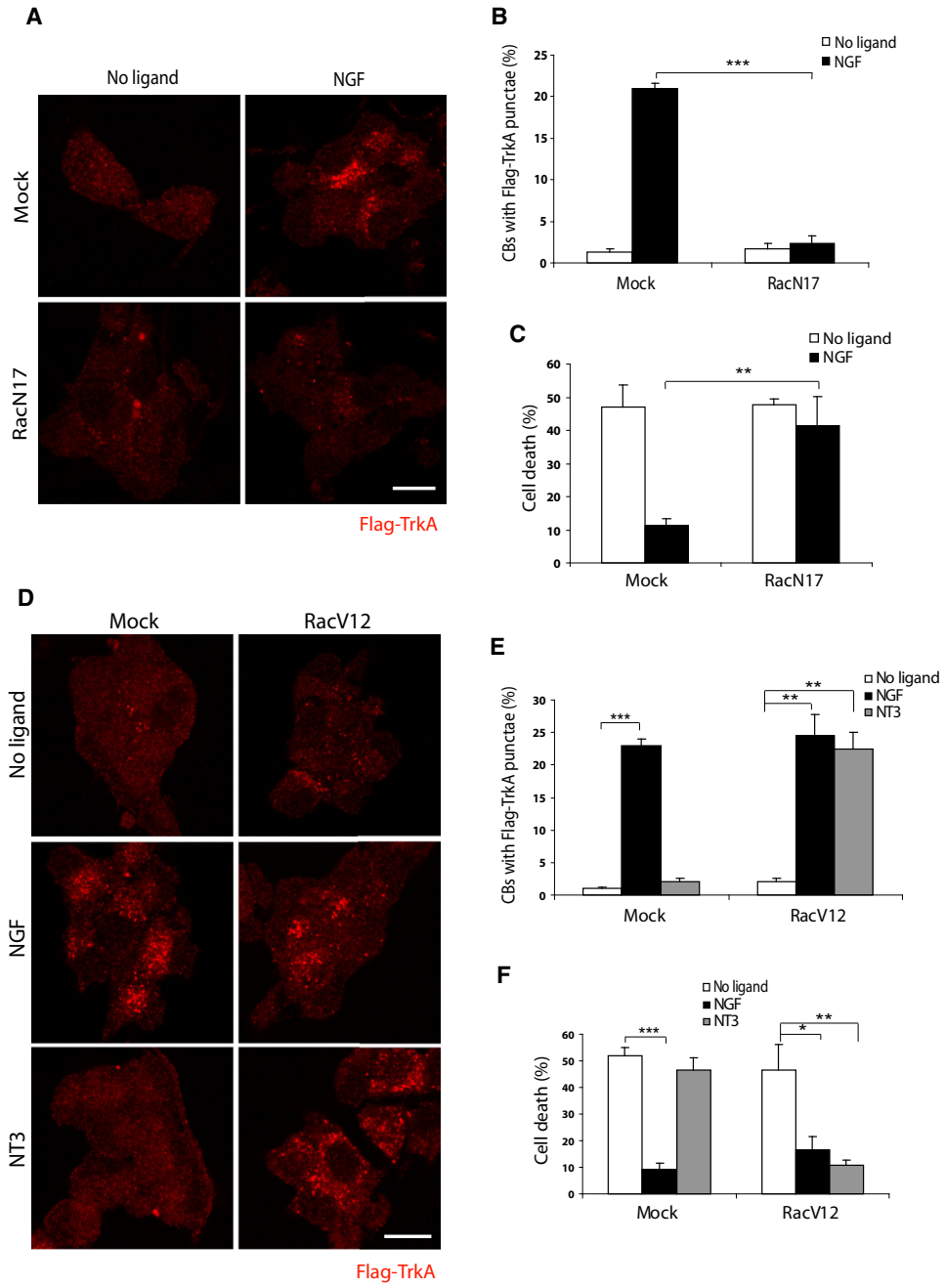

Figure 5. Rac1 Is Necessary and Sufficient for Retrograde TrkA Trafficking and Retrograde Survival

(A) Compartmentalized sympathetic neurons were infected with control or RacN17 virus and subjected to the Flag-TrkA transport assay in the presence or absence of NGF for 3 hr. Confocal images of cell bodies are shown.

(B) Percentage of cell bodies containing Flag-TrkA punctae following distal axon incubation with vehicle or NGF.

(C) Sympathetic neurons were grown as above and infected with a control or RacN17 virus. Neuronal survival was measured.

(D) Compartmentalized sympathetic neurons were infected with a control virus or a virus encoding a constitutively active form of Rac1 (RacV12). Neurons were subjected to the Flag-TrkA transport assay and stimulated on distal axons for 3 hr with vehicle, NGF, or NT3. Confocal images of cell bodies are shown.

(E) Percentage of cell bodies containing Flag-TrkA punctae following distal axon stimulation with vehicle, NGF, or NT3.

(F) Compartmentalized neurons were infected with control or RacV12 virus and were treated as indicated, and neuronal survival was measured.

Scale bars represent 10 μm. All graphical data are presented as mean ± SEM. ***$p < 0.0001$, **$p < 0.01$, *$p < 0.05$. Statistical analysis was done using one-way ANOVA followed by Tukey's post-hoc test.

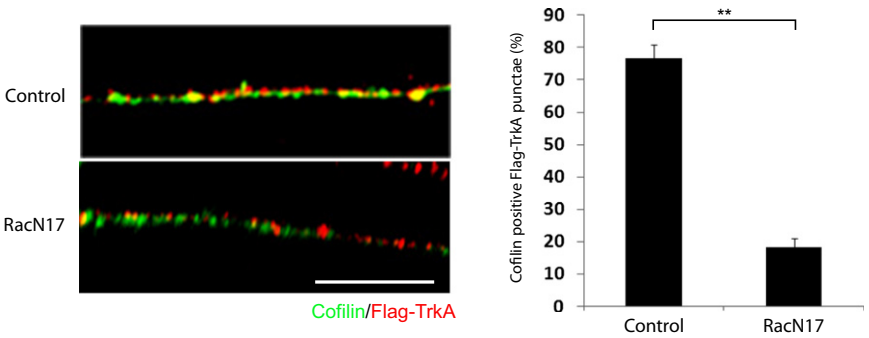

Figure 6. Rac1 Activation Is Required for Localization of Cofilin to Signaling Endosomes

Sympathetic neurons were grown in microfluidic chambers and infected with a RacN17 virus or a control virus. Two days later, neurons were subjected to the Flag-TrkA labeling assay in the presence of NGF (100 ng/ml). The effect of RacN17 on cofilin localization was examined by immunocytochemistry; internalized Flag-TrkA endosomes are labeled red and cofilin is labeled green. The percentage of cofilin-positive Flag-TrkA endosomes was quantified. Shown are means ± SEM (n = 5). Scale bars represent 10 μm. Statistical analysis was done using Student's t test. **$p < 0.01$.

in compartmentalized microfluidic chambers. Expression of RacV12 did not affect retrograde transport of Flag-TrkA endosomes following NGF treatment of distal axons. Remarkably, application of NT3 to distal axons of RacV12-expressing neurons resulted in retrograde accumulation of Flag-TrkA (Figure 5D) to an extent comparable to that seen in NGF-treated neurons (Figure 5E). Moreover, expression of RacV12 in sympathetic neurons enabled NT3 to support retrograde survival (Figure 5F). Thus, Rac1 is essential for retrograde NGF/TrkA trafficking and retrograde survival signaling, and the inability of NT3/TrkA endosomes to activate Rac1 accounts for their failure to support retrograde TrkA survival signaling.

To determine whether Rac1 activation mediates TrkA endosome formation or maturation, we asked whether it is required for internalization of the NGF/TrkA complex. We found that TrkA internalization is unaffected by RacN17 (Figure S6A). These findings are consistent with the observations that NT3, which does not activate Rac1 (Figure 3), promotes TrkA internalization (Figure 1D; Figures S1B and S1C). Thus, like cofilin, Rac1 functions at a step post-internalization and most likely during maturation of transport-competent NGF/TrkA signaling endosomes. In further support of this idea, inhibition of dynamin, a molecule required for scission of early endosomes from the plasma membrane, resulted in attenuation of the levels of Rac1-GTP following NGF treatment (Figures S6B and S6C). Thus, internalization of TrkA is necessary but not sufficient for Rac1 activation.

Rac1 Activation Mediates Cofilin Colocalization with Signaling Endosomes

NGF activates Rac1 and promotes the association of both Rac1-GTP and cofilin with TrkA endosomes, whereas NT3 does not. Moreover, Rac1 and cofilin are both dispensable for TrkA internalization; however, they are required for NGF/TrkA signaling endosome maturation, retrograde TrkA endosome transport, and retrograde survival. Whereas cofilin catalyzes actin disassembly, Rac1 is implicated in the control of both actin assembly and disassembly depending on cell type and context (Hall, 2005). What is the basis of the physical and functional relationship between Rac1 and cofilin in the context of the TrkA signaling endosome? In neutrophils and platelets, Rac1 is necessary for cofilin activation, actin depolymerization, and actin-free barbed end formation (Pandey et al., 2009; Sun et al., 2007). To ask whether Rac1 also functions upstream of cofilin in the context of TrkA signaling endosomes in sympathetic neurons, we tested the requirement of Rac1 activity for the colocalization of cofilin and NGF/TrkA endosomes. To do this, the extent of colocalization between cofilin and Flag-TrkA endosomes in distal axons of sympathetic neurons was quantified for control and RacN17-expressing neurons. Interestingly, RacN17 prevented NGF-dependent colocalization of cofilin with TrkA endosomes in distal axons (Figure 6), indicating that Rac1 initiates a signaling pathway that leads to colocalization of cofilin and NGF/TrkA endosomes. Taken together, these findings indicate that NGF, but not NT3, activates a TrkA–Rac1–cofilin signaling module that is associated with the TrkA endosome and that mediates F-actin severing and the formation of retrograde transport-competent TrkA signaling endosomes.

Differential Sensitivity to Endosomal Acidification Accounts for Differences in NGF/TrkA and NT3/TrkA Endosomes and Retrograde Survival Signaling

Why is NT3 unable to promote the formation of TrkA endosomes associated with the actin modulators Rac1-GTP and cofilin, retrograde TrkA trafficking, and retrograde survival signaling? One possibility is that the NGF/TrkA and NT3/TrkA complexes are differentially labile following endocytosis, and continual ligand–receptor association within the context of TrkA endosomes may be essential for the maturation of transport-competent signaling endosomes. We postulated that the stability of neurotrophin–TrkA interactions within the acidic environment of the early endosome (Overly and Hollenbeck, 1996) determines the state of activity of endosome-associated TrkA receptors. Indeed, differential pH sensitivities of ligand–receptor interactions have been described for EGF family ligands and their receptors (Roepstorff et al., 2009). On the cell surface, TrkA binds with higher affinity to NGF than to NT3; it is conceivable that NT3 dissociates more readily from TrkA in the lower pH environment of the early endosome, rendering it "inactive" with respect to TrkA signaling. To test this idea, we first asked whether NGF and NT3 exhibit differential pH sensitivity for TrkA activation. PC12 cells were incubated with media buffered to different pH values ranging from pH 5.5 to 7 and stimulated with either NGF or NT3. Cell lysates were subjected to immunoblotting for the phosphorylated, active form of TrkA (P-TrkA). Interestingly, although NGF and NT3 similarly stimulated TrkA phosphorylation at pH 7, NGF but not NT3 promoted phosphorylation of TrkA at

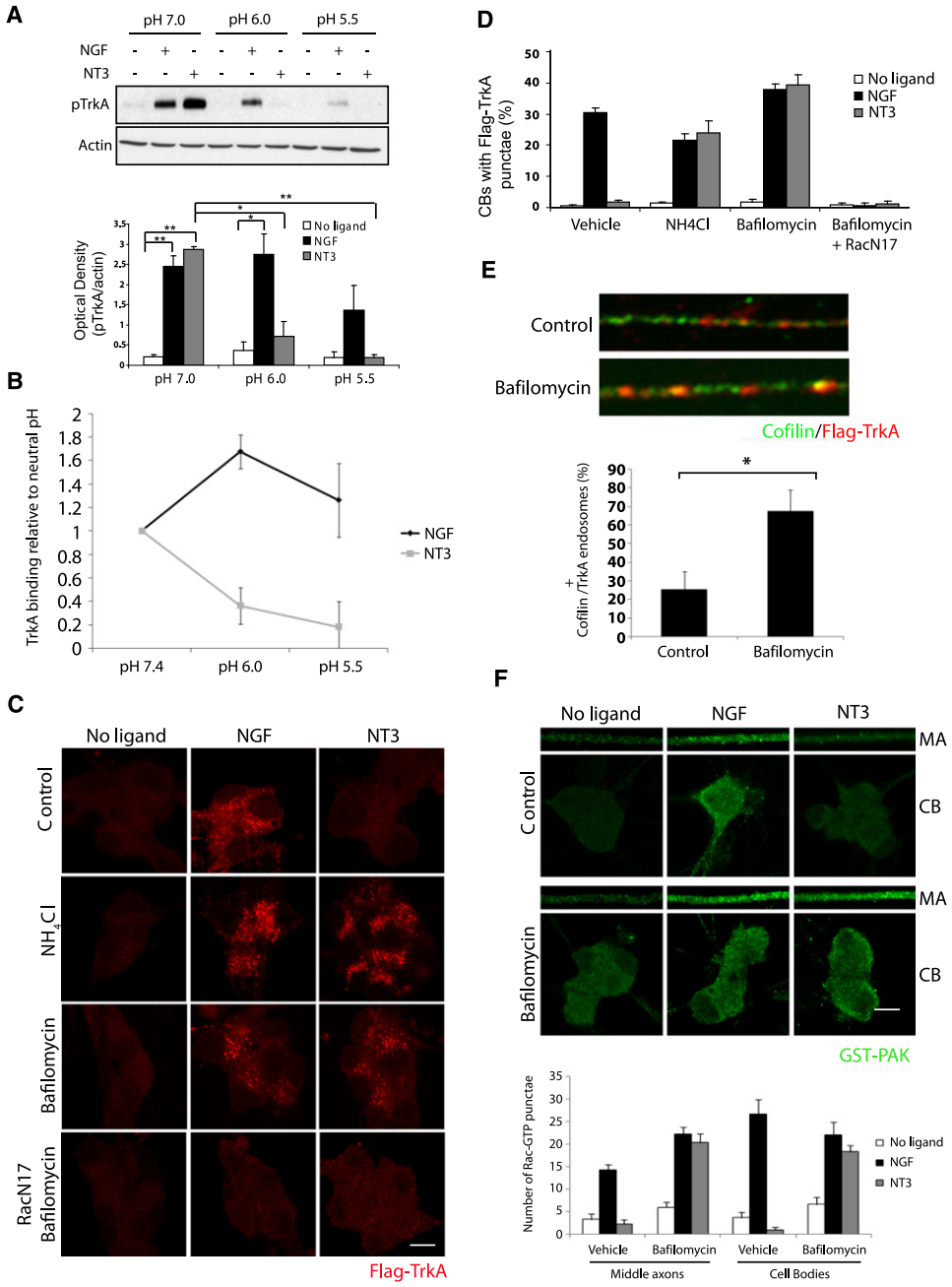

Figure 7. Blocking Endosome Acidification Enables NT3-Mediated Retrograde Transport

(A) PC12 cells were stimulated with NGF or NT3 for 10 min in DMEM buffered at pH 7.0, pH 6.0, or pH 5.5. Cells were subsequently lysed and subjected to immunoblotting for TrkA phosphorylated on tyrosine 490 (P-TrkA). Densitometric analysis was performed and P-TrkA levels were normalized against the amount of actin.

(B) Radioligand binding assays were performed in which COS7 cells in 24-well plates, either untransfected or transfected with full-length TrkA, were incubated with 1 nM ^{125}I-NGF or ^{125}I-NT3 in media buffered to the pH values indicated. Data are represented as mean ± SEM, (n = 3). Similar results were obtained in experiments using 0.2 nM ^{125}I-NGF and ^{125}I-NT3 (not shown).

(C) Compartmentalized sympathetic neurons were NGF-deprived overnight, and the Flag-TrkA transport assay was performed. Distal axons were stimulated with NGF or NT3 alone or with NGF or NT3 in the presence of NH_4Cl or bafilomycin for 3 hr. Similar experiments were done using neurons that were infected with either a control or RacN17 virus.

(D) Quantification of (C).

(E) Colocalization of internalized Flag-TrkA endosomes (red) and cofilin (green) was assessed by immunocytochemistry of distal axons of sympathetic neurons treated for 20 min with NT3 in the presence of either DMSO (vehicle control) or bafilomycin. The number of cofilin-positive Flag-TrkA endosomes in distal axons was quantified.

pH 6 and below (Figure 7A). To ask whether this difference is due to an inability of NT3 to bind to TrkA at low pH, we next performed radiolabeled neurotrophin-binding assays using media buffered at different pH values. ^{125}I-labeled neurotrophins were incubated with COS cells transfected with an expression vector encoding full-length TrkA or control vector-transfected COS cells, which served as a measure of nonspecific binding. Comparable amounts of ^{125}I-NGF bound to TrkA at pH 7.4, 6.0, and 5.5 (Figure 7B). In dramatic contrast, the ability of ^{125}I-NT3 to bind to TrkA was markedly sensitive to acidic conditions (Figure 7B). ^{125}I-NT3 bound strongly to TrkA at neutral pH, but binding of ^{125}I-NT3 to TrkA at pH 5.5 was nearly undetectable. Thus, NGF and NT3 differ in their abilities to bind and activate TrkA under the low pH conditions typically found within early endosomes.

We next asked whether the differential pH sensitivity of TrkA activation by NGF and NT3 accounts for differences in signaling endosome formation and signaling. We thus determined whether prevention of endosome acidification enables NT3/TrkA endosomes to activate Rac1, form mature Rac1-GTP and cofilin-associated endosomes, and support their retrograde transport to cell bodies. Distal axons of compartmentalized *TrkA^{Flag}* sympathetic neurons were incubated with ammonium chloride, which becomes ion-trapped within intracellular organelles and prevents their acidification, or bafilomycin, a pharmacological inhibitor of the vacuolar H^+-ATPase (V-ATPase), a pump that mediates acidification of early endosomes (Marshansky and Futai, 2008) and that we identified as a TrkA signaling endosome component (Table S1). The Flag-TrkA retrograde transport assay was then performed in neurons in which distal axons were treated with either NGF or NT3. Remarkably, following addition of either ammonium chloride or bafilomycin to distal axons, NT3 promoted retrograde transport of TrkA endosomes to cell bodies comparably to NGF (Figures 7C and 7D). Moreover, NT3 promoted colocalization of cofilin with the TrkA endosome (Figure 7E) and activation of Rac1 (Figure 7F) in neurons in which the V-ATPase was inhibited. Additionally, expression of the dominant-negative RacN17 abolished retrograde transport of TrkA induced by NGF and NT3 in the presence of bafilomycin (Figure 7D). Taken together, these findings support a model in which NGF/TrkA endosomes employ a TrkA–Rac1–cofilin signaling pathway that mediates actin severing, a necessary step for the maturation of early endosomes into retrograde transport-competent signaling endosomes. Moreover, NGF and NT3 differ in their capacities to produce Rac1-GTP/cofilin-containing endosomes and, thus, their abilities to support retrograde survival signaling due to their differential sensitivities to endosomal acidification. NT3/TrkA complexes are labile within the context of early endosomes, and as a result, mature, transport-competent NT3/TrkA endosomes fail to form. On the other hand, NGF/TrkA complexes are stable under the acidic conditions of the early endosome, enabling endosomal recruitment of Rac1-GTP and cofilin, severing of F-actin, and maturation of TrkA endosomes that are retrogradely transported to the soma.

DISCUSSION

Here, we demonstrate that the NGF/TrkA endosome is intimately associated with the actin cytoskeleton and that endosome constituents control actin severing, which is an obligate step for maturation of transport-competent signaling endosomes. Moreover, NGF/TrkA endosomes, but not NT3/TrkA endosomes, are differentially associated with key modulators of the actin cytoskeleton, explaining why NGF/TrkA endosomes are transported retrogradely to the cell body to support survival whereas NT3/TrkA endosomes are not. Our findings support a model in which the actin cytoskeleton imposes a physical barrier that restricts maturation of Rab5$^+$ TrkA early endosomes into retrograde transport-competent signaling endosomes, and the actin-severing activity associated with NGF/TrkA early endosomes is essential for overcoming this barrier. Finally, we found that NGF and NT3 differ in their capacities to support formation of mature, transport-competent TrkA endosomes associated with actin modulators because of the differentially labile nature of the neurotrophin-TrkA interactions within early endosomes. These findings define a role for actin modulation in the control of TrkA endosome maturation and signaling, and they explain how NGF produced by final target fields is the sole neurotrophin responsible for retrograde survival of developing sympathetic neurons.

Endosomal Signals Control Actin Dynamics

How do NGF/TrkA endosomes promote actin disassembly during their maturation into transport-competent signaling endosomes? We found that active forms of cofilin and Rac1 are associated with and required for retrograde transport of NGF/TrkA endosomes. Moreover, both Rac1 and cofilin are necessary for neuronal survival when NGF is acting exclusively on distal axons, but not for survival signaling in response to NGF applied directly to cell bodies. Thus, Rac1 and cofilin are essential for propagation of retrograde signaling and not TrkA survival signaling. Furthermore, Rac1 inhibition and TrkA endosome/cofilin colocalization experiments place Rac1 upstream of cofilin. Therefore, an early endosome-associated TrkA–Rac1–cofilin–actin severing signaling module is required for NGF/TrkA endosome maturation and retrograde transport.

The precise temporal and spatial patterns of activation of Rac1 and cofilin are likely crucial to their functions. We found that Rac1-GTP and cofilin are both required at a post-endocytic step for the formation of transport-competent TrkA endosomes. Recent findings in HeLa cells suggest that activation of Rac1 follows, and likely requires, clathrin-mediated endocytosis of receptor tyrosine kinases (Palamidessi et al., 2008). Consistent with this, we found that Rac1 copurifies with TrkA endosomes

(F) Distal axons of compartmentalized sympathetic neurons were stimulated with NGF or NT3 in the presence or absence of bafilomycin for 3 hr. Neurons were subsequently fixed and subjected to the GST-PAK immunoassay to visualize Rac1-GTP punctae. Quantification represents number of Rac1-GTP punctae per cell body or per 30 μm of axon.

Scale bars represent 10 μm. All graphical data shown are presented as means ± SEM. Statistical analysis was done using Student's t test (E) or one-way ANOVA followed Tukey's post-hoc test (A). $^{**}p < 0.01$, $^{*}p < 0.05$. See also Figure S7.

and that PAK-binding sites are associated with Flag-TrkA$^+$ endosomes, whereas little or no PAK binding is associated with the plasma membrane. Moreover, inhibition of TrkA endocytosis in sympathetic neurons using either a dominant-negative form of dynamin or the dynamin inhibitor, dynasore, prevented Rac1 activation (Figures S6B and S6C). Conversely, inhibition of Rac1 did not prevent internalization of TrkA. Thus, TrkA internalization is required for NGF-dependent production of Rac1-GTP, which is tethered to the NGF/TrkA endosome. On the other hand, internalization of TrkA is not sufficient for Rac1 activation because NT3 promotes formation of TrkA endosomes that are devoid of Rac1-GTP. Our results showing a requirement of Rac1 for TrkA signaling endosome maturation and retrograde transport are in agreement with previous studies in which perinuclear accumulation of TrkA following NGF treatment in PC12 cells (Valdez et al., 2007) and EGF-dependent retrograde accumulation of an EGFR-TrkB chimeric receptor expressed in sympathetic neurons (Philippidou et al., 2011) were blocked by RacN17. Thus, activation of Rac1 is essential for trafficking of Trk signaling endosomes.

Several questions remain as to how Rac1-GTP promotes recruitment of cofilin to the NGF/TrkA early endosome and how cofilin phosphorylation and activity are controlled. This is potentially broadly relevant because in other cell types, Rac1 is also required for dephosphorylation of cofilin Ser3 following receptor activation (Pandey et al., 2009; Sun et al., 2007). In at least one known case, Rac1 promotes activation of the cofilin phosphatase, Slingshot (SSH), leading to dephosphorylation of cofilin Ser3 and catalytic activation (Kligys et al., 2007). These findings also implicate a relief to inhibition of cofilin activity triggered by SSH and mediated by the binding of 14-3-3 to cofilin. Interestingly, our proteomic analysis identified both SSH and several isoforms of 14-3-3 proteins as TrkA signaling endosome-associated proteins (Table S1). We propose that Rac1 controls the actin-severing activity of the NGF/TrkA endosome and actin depolymerization by endosomal recruitment and activation of cofilin, possibly through a mechanism involving SSH and 14-3-3 proteins.

Differential Effects of NGF and NT3 on TrkA-Mediated Transport and Survival

Although both NGF and NT3 promote TrkA autophosphorylation, activation of TrkA signaling events, TrkA-dependent axonal extension, and, when applied directly to cell bodies, neuronal survival, remarkably NT3 is completely incapable of supporting retrograde transport of TrkA and retrograde survival of sympathetic neurons. In contrast to previous findings (Kuruvilla et al., 2004), our results from experiments using the highly sensitive Flag-TrkA transport assay showed that NT3 does lead to internalization of Flag-TrkA within distal axons, to a similar extent as NGF. Given that prior to visualization of Flag-TrkA endosomes, the neurons were treated with a salt-acid wash to remove cell-surface Flag antibody, the anti-Flag-labeled punctae detected in these experiments represent TrkA endosomes that have undergone a sufficient degree of endocytosis as to render them invulnerable to the surface antibody stripping conditions. Interestingly, the NT3/TrkA complexes that are internalized associate with the early endosome protein Rab5, but they are neither associated with Rac1-GTP or cofilin nor retrogradely transported to cell bodies. It is possible that NT3/TrkA is incapable of activating pathways required for either late stages of endocytosis, such as scission or translocation, or maturation of Rab5$^+$ early endosomes into endosomes that associate with the microtubule transport machinery. In any case, NT3/TrkA complexes internalize but fail to form transport-competent endosomes associated with actin cytoskeleton modulators that enable endosome maturation and long-range retrograde survival.

The pH-Sensitive Nature of NT3-Mediated TrkA Signaling

How do NGF and NT3 acting on the common receptor TrkA lead to differential activation of the Rac1-GTP–cofilin–actin signaling module? Our findings suggest that a key difference between NGF/TrkA and NT3/TrkA endosomes is the diminished ability of NT3 to bind to and support TrkA activity immediately following early endosome formation and acidification. We found that NT3 is incapable of activating TrkA and displays markedly reduced binding to TrkA at pH values below 7.0. Conversely, NT3 is capable of supporting the formation of TrkA endosomes associated with modulators of the actin cytoskeleton and retrograde transport of TrkA endosomes under conditions in which endosome acidification is prevented. We thus propose that differential maintenance of TrkA signaling within the context of the early endosome accounts for the distinct roles of NT3 and NGF during sympathetic neuron development (Figure S7). The labile nature of NT3/TrkA complexes following endosome formation and acidification ensures a transient, local mode of action of NT3. This accounts for NT3's ability to support axonal extension but not retrograde survival (Kuruvilla et al., 2004) (Figure 1A). In contrast, acid-stable NGF/TrkA complexes promote activation of an endosomal TrkA–Rac1–cofilin–actin signaling module, which enables maturation of transport-competent signaling endosomes that propagate retrogradely to the cell body where they support survival and the formation of synapses with preganglionic partners. Interestingly, the ability of NT3 to promote retrograde transport of TrkA and retrograde survival in neurons expressing a constitutively active Rac1 suggests that there may be sufficient ligand-receptor engagement under acidic endosomal conditions to maintain the low levels of downstream signaling in the cell body that are necessary for survival. An alternative possibility is that RacV12 may enable survival signaling from NT3-formed endosomes. Ultimately, the differential sensitivity to endosomal pH accounts for the unique ability of NGF, and not NT3, to establish proper matching between the size of the neuronal population and the size and demands of the target field.

In summary, our findings indicate that NGF/TrkA endosomes employ a signaling pathway composed of Rac1 and cofilin that directs the breakdown of F-actin, a necessary step for maturation of retrograde transport-competent TrkA signaling endosomes. Moreover, the formation of TrkA endosomes associated with Rac1 and cofilin represents a divergence point for NGF and NT3 signaling in sympathetic neurons. We propose that differential activation of endosomal signaling pathways that culminate in actin severing and relief of an "actin block" on maturation of transport-competent TrkA signaling endosomes accounts for the differences between the local axon-growth-promoting effects of the

intermediate target-derived factor, NT3, and the long-distance, retrograde-mediated effects of final target-derived NGF.

EXPERIMENTAL PROCEDURES

Sympathetic Neuron Culture and the Flag-TrkA Endosome Transport Assay

Sympathetic neurons from mouse SCGs were cultured as previously described (Deppmann et al., 2008). The Flag-TrkA endosome transport assay was performed as described previously (Sharma et al., 2010). Briefly, an anti-Flag antibody was added to distal axons of neurons plated in microfluidic chambers at 4°C. After washing out unbound antibody, neurons were moved to 37°C for 3 hr. Surface TrkA signal was stripped with a 0.5 M NaCl/0.2 M acetic acid solution, and neurons were fixed and stained with an anti-mouse fluorescent secondary.

Sympathetic Neuron Cultures in Microfluidic Chambers

Microfluidic chambers were produced as described in Park et al. (2006) and fixed to coverglass coated with poly D-lysine (50 μg/ml) and laminin (1 μg/ml). Dissociated neurons seeded in chambers were grown for 3 to 5 days in vitro (DIV) to allow axons to project into the distal chamber. Total volume differential between the two compartments was maintained at 100 μl to ensure fluidic isolation during experiments.

Actin Disassembly Assay

Purified nonmuscle actin (Cytoskeleton, Inc.) was polymerized in F-buffer (5 mM Tris-HCl [pH 8.0], 0.2 mM $CaCl_2$, 50 mM KCl, 2 mM $MgCl_2$, and 1 mM ATP). F-actin (2 μM) was then incubated with TrkB/A endosome-coated Dynabeads or control Dynabeads at room temperature for 15 min. Addition of 4 μM DBP (Sigma) initiated the actin disassembly reaction. At 15 min following DBP addition, beads were magnetically removed from samples and polymerized, and monomeric actin was separated by ultracentrifugation at 100,000 × *g* for 20 min. Protein was visualized by SDS-PAGE and Coomassie staining.

Sympathetic Neuron Survival Assay

To assess retrograde survival, anti-NGF antibody was present within the cell body/proximal axon compartments of microfluidic chamber devices while NGF or NT3 was added to the distal axons. After 36–40 hr of treatment, cells were washed, fixed with 4% paraformaldehyde, washed, and stained with Hoechst 33258 (2 μg/ml; Invitrogen). A neuron was scored as apoptotic if it contained no nucleus or a nucleus that was fragmented or condensed. A minimum of 100 neurons was scored per condition.

Cell Fractionation for Immunoblot, Electron Microscopy, and Mass Spectroscopy

A detailed procedure for the purification of TrkA endosomes and their preparation for immunoblot, electron microscopy and mass spectroscopy can be found in the Extended Experimental Procedures.

GST-PAK Immunoassay and Pull-Down Assay

Sympathetic neurons were grown in either mass culture or microfluidic chambers, treated as indicated, and fixed for 10 min using 4% paraformaldehyde. Cells were then washed and blocked in PBS containing 1% BSA/0.1% Triton X-100 for 30 min at room temperature. This was followed by incubation with a solution containing 5 μg/ml GST-PAK-PBD for 4 hr to overnight at 4°C. Next, cells were washed and incubated with anti-GST-Alexa 488 secondary antibody for 30 min at room temperature, mounted, and examined using confocal microscopy to visualize sites of Rac1-GTP. For pull-down assays, PC12 6-24 cells that overexpress TrkA (Stephens et al., 1994) or sympathetic neurons from three litters of WT mice were grown for 2 DIV and serum-deprived (PC12 cells) or serum- and NGF-deprived with anti-NGF and BAF (sympathetic neurons) for 16 hr. Cells were treated as described in the legends and pull-downs were done as described in Extended Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, seven figures, and one table and can be found with this article online at doi:10.1016/j.cell.2011.07.008.

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